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A Better Path™

Chapter 16

Troubleshooting

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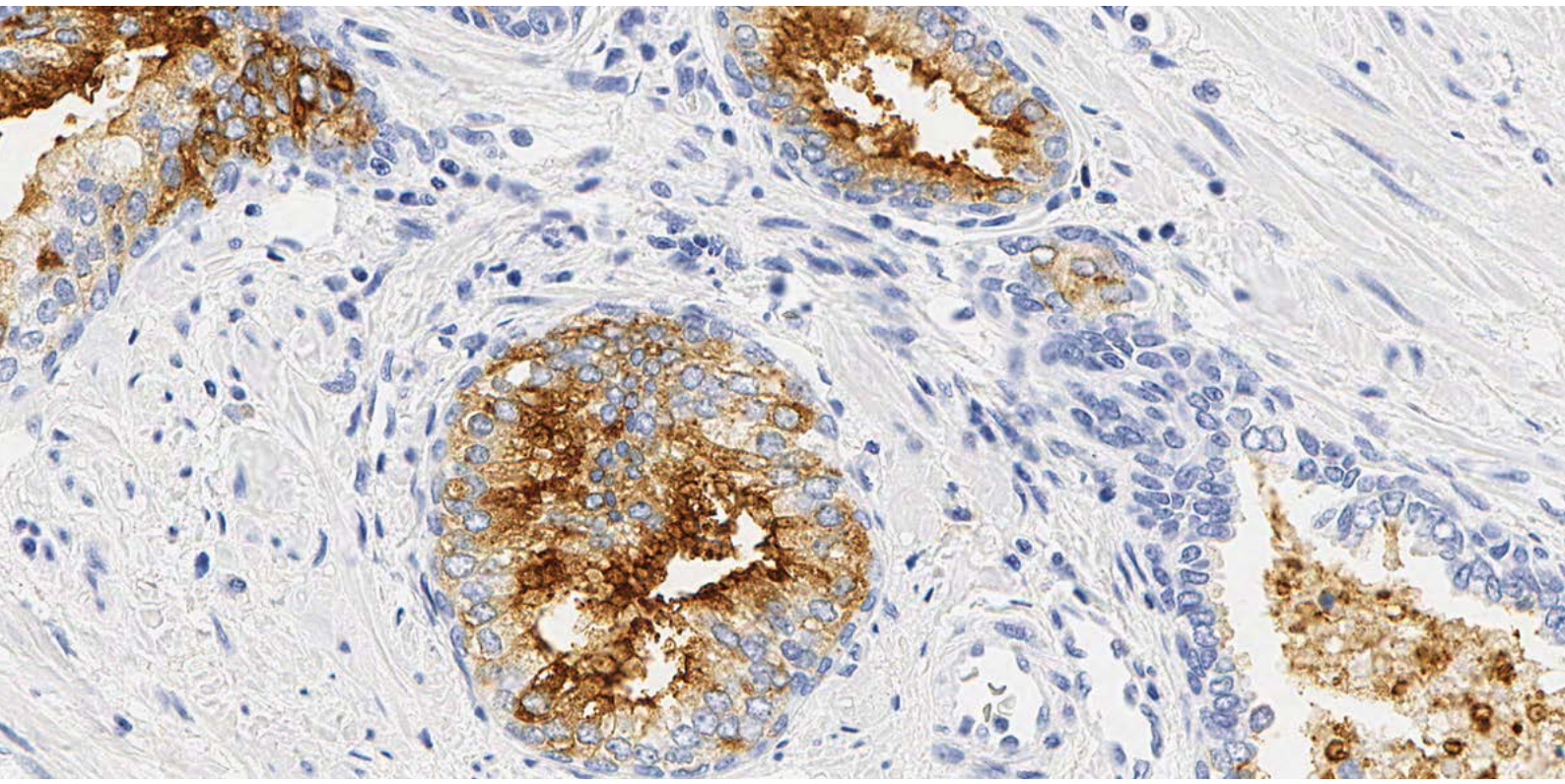

Trou•ble•shoot•ing *(n.)*
Discovering why something does not work effectively and making suggestions about how to improve it.

Cambridge Advanced Learner's Dictionary

Chapter 16.1 | Introduction

Immunohistochemistry (IHC) is a multi-step process that requires specialized training in the processing of tissue, the selection of appropriate reagents and interpretation of the stained tissue sections. In general, IHC staining techniques allow for the visualization of antigens by sequential application of a specific antibody to the antigen, a secondary antibody to the primary antibody, an enzyme complex and a chromogenic substrate. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. Because of its highly complex nature, the causes of unexpected negative reactions, undesired specific staining, or undesired background may be difficult to isolate. The information contained in this chapter should enable the user rapidly to pinpoint and resolve problems encountered during the staining procedure.

Section 1 is a compilation of common problems encountered when using IHC staining reagents, including the underlying causes of staining failure and the recommended corrective actions. The chart is divided into sections describing inadequate staining, general background staining, and limited background staining.

Section 2 presents a method of systematically adding one IHC reagent at a time to determine at which stage in a staining protocol non-specific or undesired staining may be occurring.

Section 3 is a simple chart used to define the type of tissue specimen, the IHC reagents, and the staining protocol already in use by the laboratory personnel. The user is encouraged to copy this chart and use it to help troubleshoot any problems that may be encountered in the IHC staining process.

Section 4 is a guide to reading a manufacturers' specification sheet for IVD (in vitro diagnostic) antibodies. The guide includes general information for use in immunohistochemistry, including fixation, recommended visualization systems, recommended titer and diluent, pre-treatment methods, and selection of required controls.

Section 5 is a guide to check that the automated platform used to perform the staining has operated correctly during the staining process.

Section 1 | Common Problems

Inadequate Staining (little or no specific staining)

Possible cause of poor staining	Solution
A) Both the positive controls and the specimen tissue show little or no specific staining, except for counterstain. The tissue section may show little or no background staining.	
Primary antibody or labeled reagent omitted. Reagents used in wrong order.	Repeat the procedure using the manufacturer's staining system specification sheet, or the standard operating procedure reagent checklist as established by the individual laboratory.
Excessively diluted or excessively concentrated reagents; inappropriate incubation time and or temperature.	Determine correct concentration for each reagent (see **Chapter 4** and **Chapter 5**). Depending on the degree of staining obtained, if any, a 2- to 5- fold increase in concentration may be needed. Incubation temperature and incubation time are inversely proportional and will affect results. To determine optimal incubation protocol, vary either the time or temperature for each reagent in the IHC staining system. Generally, incubation times can be extended if little or no background is detected. Overnight incubation at higher dilution may also be effective.
Primary antibody diluted with inappropriate buffer. Use of PBS or TBS as an antibody diluent. Lack of stabilizing or carrier protein. Detergent in diluent.	Check formula and compatibility of antibody diluent. A change of ion content and/or pH of the antibody diluent can cause a diminution in the sensitivity of the antibody. Addition of NaN_3 should be avoided. This problem is primarily seen with monoclonal antibodies.

Possible cause of poor staining	Solution
Primary antibody defective; one or several secondary or ancillary reagents defective. Do NOT use product after expiration date stamped on vial.	Replace defective or expired antibody; repeat staining protocol, replacing one reagent at a time with fresh, in-date reagents. ■ Store products according to each product specification sheet or package insert ■ If using a neat or concentrated antibody, and directed by the manufacturer to store frozen, the reagent may be aliquoted to avoid repeated freezing and thawing ■ Do not freeze ready-to-use (RTU) or customer diluted products ■ Follow manufacturer recommendations on product specification sheets, package inserts, and reagent labels
Dissociation of primary antibody during washing or incubation with link antibodies.	Particularly a feature of low affinity antibodies: ■ Polyclonal primary antiserum: Attempt staining at lower dilutions (higher concentrations) ■ Monoclonal primary antibody: Replace with higher affinity antibody of identical specificity ■ Re-optimize incubation times for washing buffer and link antibody
Use of alcohol-based counterstain and/or alcohol-based mounting media will remove aqueous–based chromogens.	■ Repeat staining, using water-based counterstain and mounting media ■ Use a permanent chromogen, such as DAB/DAB+, that is not affected by organic solvents
Excessive counterstaining may compromise proper interpretation of results.	Use a counterstain that: ■ Will not excessively stain tissue sections ■ Can be diluted so as not to obliterate the specific signal ■ Reduce incubation time of the counterstain
Incorrect preparation of substrate-chromogen mixture.	■ Repeat substrate-chromogen treatment with correctly prepared reagent ■ Staining intensity may be decreased when excess DAB/DAB+ is present in the working reagent
Incompatible buffer used for preparation of enzyme and substrate-chromogen reagents: Use of PBS wash buffer with an alkaline phosphatase staining system. Sodium azide in reagent diluent or buffer baths for immunoperoxidase methodologies.	Check compatibility of buffer ingredients with enzyme and substrate-chromogen reagents. Repeat staining: ■ Commercial phosphate buffers may contain additives that will inhibit alkaline phosphates activity ■ Avoid sodium azide in diluents and buffers. A concentration of 15 mM/L sodium azide, which is routinely added to IHC reagents to inhibit bacterial growth, will not impair HRP conjugated labels
Antigen levels are too low for detection by the employed visualization system. May be due to loss of antigenic differentiation in some tumors or loss of antigenicity due to sub-optimal tissue fixation.	■ Utilize a higher sensitivity staining system ■ Prolong incubation time of primary antibody ■ Re-optimize incubation times and concentrations of ancillary reagents ■ Perform antigen retrieval, if applicable, using a range of pH buffers (see **Chapter 3**)
Steric hindrance due to high antigen level and possible prozone effect.	Re-optimize concentration of the primary antibody and ancillary reagents. Antibody concentration of the primary antibody may be too high.
Use of inappropriate fixative. Use of certain fixatives may damage or destroy antigens or epitopes in the tissue specimen. Use of non-cross linking fixatives may allow the elution of antigens soluble in IHC reagents. Different fixatives may affect standardization of cells.	Check manufacturer's specifications regarding recommended fixatives known to be effective with antibody and protocol in use.
Immunoreactivity diminished or destroyed during embedding process.	Use a paraffin wax with a melting temperature ~55-58 °C. Wax used for embedding should not exceed 60 °C.
Immunoreactivity diminished or destroyed during dewaxing at high oven temperature.	Oven temperature not to exceed 60 °C. **NOTE:** The intensity of immunostaining may be diminished when tissue is exposed to prolonged heat at this stage in the protocol. Paradoxically heating of the section in aqueous solution is used to recover antigenicity in the AR process (See **Chapter 3**). Refer to the primary antibody specification sheet for additional information.
Immunoreactivity diminished or destroyed on pre-cut tissue sections.	The intensity of immunostaining may be diminished when pre-cut tissue sections are exposed to air. Use freshly cut sections and re-seal paraffin-embedded blocks.
Immunoreactivity diminished or destroyed by the enzyme blocking reagent altering a specific epitope.	More common on frozen sections: apply the primary antibody prior to the enzymatic block to ensure its reaction. In such cases the blocking reagent can be applied at any point after the primary and before the enzyme-labeled components.

Possible cause of poor staining	Solution
Excessive wash buffer or blocking serum remaining on tissue section prior to application of IHC reagents.	Excess residual reagent will dilute the next consecutive reagent. Repeat staining, making sure to wipe away excess washing buffer and blocking serum.
Antigen retrieval protocol is inappropriate or has been omitted.	Many tissue antigens require proteolytic enzyme digestion or heat-induced antigen retrieval performed prior to staining (Chapter 3). The need for pre-treatment depends on the type and extent of fixation, specific characteristics of the antigen and the type of antibody used. Use the pretreatment method recommended by the manufacturer. No single pre-treatment is suitable for all applications.
Repeated reuse of antigen retrieval buffer.	Do not reuse buffer.
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol, using fresh xylene or xylene substitute.
Failure to achieve the optimal temperature required for heat induced antigen retrieval.	■ When using a waterbath or steamer, allow sufficient time for the retrieval buffer to equilibrate to a temperature range of 95-99 °C ■ At high altitude (greater than ~4,500 feet), the buffer will boil at less than 95 °C ■ Utilize a closed heating system such as a pressure cooker, autoclave or Pascal, or utilize a low temperature protocol if standardization of the validated procedure is not affected
Excessive or incomplete counterstaining.	Re-optimize concentration of counterstain and incubation time.
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specifications.

B) Positive control tissue shows adequate specific staining with little or no background staining. Specimen tissue shows little or no specific staining with variable background staining.

Specimen held for too long in a cross-linking fixative, usually in formalin, causing "masking" of antigenic determinants. Control appropriately fixed.	Standardize routine fixation, matching test specimens to control tissues. Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive (Chapter 3). Refer to the primary antibody specification sheet for additional information.
Sectioned portion contains crush artifact caused by grossing tissue with dull scalpel or razor.	Serum proteins diffuse through tissue and are fixed in place. Cut new tissue block if available, using sharp blade.
Sectioned portion of specimen contains necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.
Uneven fixation of section; portion of specimen not penetrated by fixative. Loss of antigenicity in unfixed tissue.	Fix tissue biopsy for longer period of time or fix smaller pieces to ensure complete penetration. Unfixed tissue tends to bind all reagents non-specifically.

General Background

Background is seen in both control tissue and specimen tissue. Background staining may affect several tissue elements, such as connective tissue, adipose tissue and epithelium.

Excessive incubation with substrate-chromogen reagent.	Reduce incubation time.
Substrate-chromogen reagent prepared incorrectly.	Repeat incubation with correctly prepared chromogen reagent.
Secondary or link antibody cross-reacts with antigens from tissue specimen.	Absorb link antibody with tissue protein extract or species-specific normal serum from tissue donor.
Secondary or link antibody and/or tertiary reagents too concentrated.	Repeat staining. Determine correct concentration for each reagent. Incubation temperature and incubation time will affect results. To determine optimal incubation protocol, vary both the time and temperature for each reagent in the IHC staining protocol.
Substrate-chromogen reagent prepared incorrectly.	Repeat incubation with correctly prepared chromogen reagent.

Possible cause of poor staining	Solution
Slides inadequately rinsed.	Gently rinse slide with wash buffer bottle and place in wash bath for 5 minutes. Gentle agitation of the wash bath may increase effectiveness when used with cytoplasmic or nuclear staining protocols.
Insufficient saline or detergent in wash buffer.	High-sensitivity staining systems may require higher concentrations of saline or detergent in the wash buffer. Refer to the staining system specification sheet for optimal formulation.
Blocking serum or wrong blocking serum used.	Block with serum from the host of the secondary or link antibody. Avoid serum that contains auto-immune immunoglobulins. Alternatively, a serum-free protein block, lacking immunoglobulins, may be substituted for the serum block.
Sections incorrectly dewaxed.	Prepare new sections and deparaffinize according to standard laboratory protocol using fresh xylene or xylene substitute.
Non-specific binding of the secondary antibody with an animal tissue specimen.	Use a secondary antibody that has been absorbed against a species specimen, or use a secondary antibody produced in a host that exhibits little or no cross-reactivity with the tissue source.
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specification.
Specimen tissue and negative reagent control slides show background staining. Positive and negative control tissue show appropriate specific staining. May involve several tissue elements such as connective tissue, adipose tissue and epithelium.	
Specimen held for too long in a cross-linking fixative, usually in formalin, causing 'masking' of antigenic determinants due to aldehydes cross-linking and increased hydrophobicity of tissue.	Standardize routine fixation. Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody specification sheet for additional information.
Sectioned portion of specimen not penetrated by fixative. Loss of antigenicity in unfixed tissue. Unfixed tissue tends to bind all reagents nonspecifically.	Fix tissue biopsy for longer period of time or fix smaller pieces to ensure complete penetration.
Sectioned portion contains crush artifact caused by grossing tissue with dull scalpel or razor. Serum proteins diffuse through tissue and are fixed in place.	Serum proteins diffuse through tissue and are fixed in place. Re-cut tissue using sharp blade.
Sectioned portion of specimen contains necrotic or otherwise damaged elements.	Ignore physically damaged portions of stained tissue sections.
Excessive or unevenly applied subbing agent on poly-L-lysine, charged, or silanized slides.	Some IHC reagents may bind to these products, resulting in a light stain over the entire slide surface. Some slides may be unevenly coated, and will exhibit the above problems on only a portion of the tissue or glass.
Antigen diffusion prior to fixation causing specific background outside the expected antigen site.	Avoid delays in fixation of the tissue.
Tissue sections too thick.	Cut tissue sections thinner. Formalin-fixed paraffin-embedded tissue sections should be approximately 4-6 µm; cryostat section 4-6 µm or less.
Incomplete permeabilization of tissue sections.	Seen in frozen sections, cell smears and non-paraffin embedded tissue: incomplete permeabilization of cells allows unattached reagents to become trapped within the cells and resistant to removal by wash buffer.

Possible cause of poor staining	Solution
Negative reagent control slide shows background. Positive control tissue, negative control tissue and specimen tissue show expected specific staining.	
Negative control serum insufficiently diluted.	Use properly diluted negative reagent control serum: ■ For polyclonal antibodies, dilute the negative reagent control serum until the protein concentration is equal to that of the primary antibody ■ For monoclonal antibodies, dilute the negative reagent control serum until the Ig concentration is equal to that of the primary antibody
Contaminating antibodies in the negative control serum are cross-reacting with proteins from the specimen tissue.	Replace the negative reagent control serum; repeat staining protocol.
Negative reagent control serum contaminated with bacterial or fungal growth.	Replace product with non-contaminated serum.

Limited Background

Areas of inconsistent staining on controls, specimens and glass slides.	
Protein trapped beneath the tissue during the mounting process will allow partial lifting of the section. Pooling of IHC reagents beneath the section, or partial detachment of the tissue from the slide may occur.	Avoid the use of commercial adhesives, glue starch or gelatin in water baths when mounting tissue sections. Avoid allowing water from an initial section mounting to flow over an area where additional sections will be mounted. This is particularly important when using charged or silanized slides.
Undissolved granules of chromogen.	Ensure that chromogen in tablet or powder form is completely dissolved, or switch to a liquid chromogen.
Incomplete dezenkerization of tissue fixed with B5 or mercury containing reagents.	Remove embedding medium thoroughly, using fresh reagents.
Incomplete dezenkerization of tissue fixed with B5 or mercury containing reagents.	Perform dezenkerization with fresh reagents.
Bacterial or yeast contamination from mounting waterbath.	Clean and refill waterbath.
Partial drying of tissue prior to fixation. Unaffected areas show normal staining.	■ Immerse tissue promptly in fixative or holding reagent ■ Keep moist during the entire staining process ■ Use a humidity or moist chamber during incubation steps ■ When using an automated staining instrument, addition of wet towels to the sink may prevent drying of slides
Instrument malfunction.	Ensure automated stainer is programmed correctly and is running to manufacturer's specification.
Adipose or connective tissue in specimen, negative control tissue, positive control tissue and negative reagent control slides. Background in connective and epithelial tissue.	
Hydrophobic and ionic interactions between immunoglobulins and lipoid substances in fatty tissue.	Non-specific staining of fatty tissue rarely interferes with interpretation of specific staining and can usually be disregarded.
Primary antibody and negative reagent control serum are insufficiently diluted.	Reoptimize the dilution of the primary antibody and negative control serum.
Epithelial tissue in specimen, negative control tissue, positive control tissue and negative reagent control slides. Staining is moderate to marked, especially in epidermal epithelium. Background in epithelia accompanies background in connective tissue.	
Both the primary antibody and negative control serum contain contaminating antibodies to epithelial elements, possibly cytokeratins.	■ Use a higher dilution of the primary antibody and negative control serum ■ Increase the incubation time ■ Replace the antibody
Excessive formalin fixation of tissues may increase protein cross-linking, resulting in tissue hydrophobicity.	Proteolytic digestion or antigen retrieval will break down cross-linking and render some tissue antigens reactive. Refer to the primary antibody and/or the negative reagent control specification sheet for appropriate pre-treatment.

Possible cause of poor staining	Solution
Focal cytoplasmic staining observed in epithelium in the specimen tissue.	
Focal cytoplasmic staining is seen, particularly in intermediate and superficial layers of the epidermis. May be caused by passive absorption of plasma proteins into degenerating epidermal cells.	This observation is rare and should not interfere with interpretation of specific staining.
Background seen in all control and specimen tissue when using an immunoperoxidase staining system.	
Unquenched endogenous peroxidase activity may be seen in all hemoprotein-containing specimens, including hemoglobin in erythrocytes, myoglobin in muscle cells, cytochrome in granulocytes and monocytes and catalases in liver and kidney.	■ Use alternate or prolonged peroxidase blocks or use another enzyme label such as alkaline phosphatase ■ Eosinophils and mast cells are particularly resistant to peroxidase quenching. Use a peroxidase blocker ■ Use special stains: eosin will stain eosinophils a bright red-orange
Background seen in all control and specimen tissue when using an alkaline phosphatase staining system.	
Unquenched endogenous alkaline phosphatase activity may be seen in leucocytes, kidney, liver, bone, ovary bladder, salivary glands, placenta and gastro-intestinal tissue.	Add levamisole to the alkaline phosphatase chromogen reagent or use another enzyme label such as horseradish peroxidase. Intestinal alkaline phosphatase is not quenched by the addition of levamisole. Pretreat the tissue with 0.03 N HCl.
Background seen in all control and specimen tissue when using a biotin-streptavidin staining system.	
Endogenous protein-bound biotin (water-soluble B vitamin). High amounts of biotin are found in adrenal, liver, and kidney. Lesser amounts are found in the GI tract, lung, spleen, pancreas, brain, mammary gland, adipose tissue, lymphoid tissue, and cells grown in culture media containing biotin as a nutrient.	Use a biotin block or chose another non-biotin based staining system.
Background of skeletal or smooth muscle tissue in positive control tissue, negative control tissue, specimen tissue and negative reagent control.	
Cause is not understood. It is possibly due to antibodies to muscle antigens in primary and negative reagent control serum.	Should not interfere with interpretation of specific staining.
Undesired 'Specific' Staining. Positive staining of leucocyte membranes in specimen tissue, positive control, negative tissue control and negative reagent control.	
Binding of the Fc portion of Ig by Fc receptors on the cell membrane of macrophages, monocytes, granulocytes and some lymphocytes.	■ Use F(ab')$_2$ or F(ab) fragments for the primary and secondary antibodies rather than intact antibodies ■ Add detergent to the wash buffer
Positive staining of histiocytes and granulocytes in the specimen tissue only, with a marker not normally reactive with these cells.	
Phagocytosis of antigens may render phagocytes positive for the same.	Rare. Should not interfere with interpretation of specific staining.
Positive membrane staining of specimen tissue and negative reagent control tissue when using a horseradish peroxidase staining system.	
Tissue from persons infected with Hepatitis B virus and expressing Hepatitis B surface antigen may exhibit undesired staining.	Utilize a non-peroxidase staining system.

Miscellaneous

Loss of viability of cell cultures.	
Some manufacturers produce antibodies and reagents for in vitro use only. These products may contain preservatives, usually sodium azide, which is a known poison.	Utilize an in vivo product for application on viable cells. For use on cell cultures only: sodium azide may be dialyzed out of some reagents. Contact Dako Technical Support for additional information.

Section 2 | Systematical Approach Using a Troubleshooting Flow Chart

This flow chart can be used to determine source(s) of non-specific staining that has been encountered when using an IHC staining protocol. Each step (Slide box to the left) is a suggestion for reagents to be tested on the indicated tissue type with known positive or negative expression pattern. In the first step (Slide #1) the tissue is only counterstained with hematoxylin. If the described result (to the right) does not match the observed staining pattern when using the suggested setup, proceed to the next step in the flow chart. In the next step (Slide #2), a chromogen is added to the staining protocol, before counterstaining and so forth.

Background Staining Encountered with Peroxidase Reagents

Reagents	Result/Action
Slide 1 **Positive Control Tissue:** Counterstain with hematoxylin If Result/Action does not match the observed staining: Go to next step	Brown endogenous pigment (such as melanin) observed: ■ To distinguish melanin pigment from DAB chromogen, Azure B can be used as a counterstain. The melanin stains blue-green, while the DAB remains brown. ■ An alternate method is to use AEC as the chromogen. However, if high levels of pigment exist in the tissue, the red chromogen may be partially obscured. Since bleaching protocols to remove melanin may compromise tissue antigenicity, it should be avoided if at all possible.
Slide 2 **Positive Control Tissue:** DAB/AEC chromogen and counterstain If Result/Action does not match the observed staining: Go to next step	Brown/red color observed: ■ Indicates endogenous peroxidase activity in the tissue sections. It is present in all hemoprotein containing tissue including erythrocytes, muscle, liver, kidney, granulocytes and monocytes. ■ Block with 3% hydrogen peroxide or other peroxidase blocking reagent. Using a new bottle of hydrogen peroxide, perform a 3% H_20_2 peroxidase block, followed by DAB and an appropriate counterstain.
Slide 3 **Positive Control Tissue:** Peroxidase Block + Secondary Antibody + Streptavidin-HRP DAB/AEC Counterstain If Result/Action does not match the observed staining: Go to next step	Brown/red color observed: ■ Indicates endogenous biotin activity in the tissue sections. Protein-bound biotin may be found in adrenal, liver, kidney, GI tract, lung, spleen, brain, mammary gland, adipose tissue, lymphoid tissue and cell grown in culture media containing biotin (RPMI, NCTC, MEME). ■ Block with a biotin block or switch to a staining system that is not dependent on the streptavidin/biotin reaction

Reagents	Result/Action
Slide 4 **Positive Control Tissue:** Peroxidase Block + Biotin Block (if required) + Secondary Antibody + Streptavidin-HRP + DAB/AEC + Counterstain	Brown/red color observed: ■ Indicates non-specific or undesired binding of the secondary antibody to the tissue sections. This primarily occurs when the secondary antiserum has not been prepared for use on a specific species tissue. ■ To determine if this is the problem, absorb out non-specific proteins by adding 2, 5 or 10 µL of normal serum (from the species of tissue to be stained) per 100 µL of the secondary antibody
If Result/Action does not match the observed staining: Go to next step ▼	
Slide 5 **Positive Control Tissue:** Peroxidase Block + Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-HRP + DAB/AEC	Brown/red color observed: ■ May indicate non-specific binding of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody. Add 0.05-0.1% Tween 20 to wash buffer to decrease protein attachment. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections
If Result/Action does not match the observed staining: Go to next step ▼	
Slide 6 **Negative Control Tissue:** Perform complete staining protocol	Brown/red color observed on Negative Control Tissue: ■ Monoclonal antibody: Possible contamination ■ Polyclonal antibody: Possible contamination or undesired antibody in the host Ig fraction ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections

Background Staining Encountered with Alkaline Phosphatase

Slide 1 **Positive Control Tissue:** Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/blue color observed: ■ Indicates endogenous alkaline phosphatase activity in the tissue sections. It is present in liver, kidney, GI tract, bone, bladder, ovary, salivary gland, placenta, leukemic, necrotic or degenerated cells. ■ Block with levamisole (Intestinal alkaline phosphatase may be quenched by the addition of 0.03 N HCl prior to the addition of the alkaline phosphatase)
If Result/Action does not match the observed staining: Go to next step ▼	

Reagents	Result/Action
Slide2 **Positive Control Tissue:** Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/Blue color observed: ■ Indicates endogenous biotin activity in the tissue sections. Protein-bound biotin may be found in adrenal, liver, kidney, GI tract, lung, spleen, brain, mammary gland, adipose tissue, lymphoid tissue and cells grown in culture media containing biotin (RPMI, NCTC, MEME). ■ Block with a biotin block or switch to a staining system that is not dependent on the streptavidin/biotin reaction
If Result/Action does not match the observed staining: Go to next step ▼	
Slide 3 **Positive Control Tissue:** Biotin Block (if required) + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain	Red/blue color observed: ■ Indicates non-specific or undesired binding of the secondary antibody to the tissue sections. This primarily occurs when the secondary antiserum has not been prepared for use on a specific species tissue. ■ To determine if this is the problem, absorb out non-specific proteins by adding 2, 5 or 10 µL of normal serum (from the species of tissue to be stained) per 100 µL of the secondary antibody
If Result/Action does not match the observed staining: Go to next step ▼	
Slide 4 **Positive Control Tissue:** Biotin Block (if required) + Negative Reagent Control + Secondary Antibody + Streptavidin-AP + Fast Red, Fuchsin or BCIP/NBT + Counterstain.	Red/blue color observed: ■ May indicate non-specific binding of the primary antibody carrier-protein. Perform a protein block with normal serum from the host of the link antibody or a protein block; add 0.05-0.1% TWEEN 20 to wash buffer to decrease protein attachment. ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue or as specific staining in pancreatic sections
If Result/Action does not match the observed staining: Go to next step ▼	
Slide 5 **Negative Control Tissue:** Perform complete staining protocol	Red/blue color observed on Negative Control Tissue: ■ Monoclonal antibody: Possible contamination ■ Polyclonal antibody: Possible contamination or undesired antibody in the host Ig fraction ■ Antigen retrieval lipofusion-artifact may appear as granule staining in liver and cardiac tissue, or as specific staining in pancreatic sections

Negative Reagent Control

Reagents

Negative Control Reagent:
Perform complete staining protocol.

Result/Action

- (Human tissue) Perform the peroxidase blocking protocol from Slide #2 under "Background Staining Encountered with Peroxidase Reagents"
- Perform a biotin block if required, protein block if required, apply the appropriate negative reagent control (see below), apply biotinylated secondary antibody, apply streptavidin/HRP reagent and DAB
- Prepare a negative reagent control
  - Polyclonal: non-immunized sera from the same species, diluted to the same protein concentration as the primary antibody
  - Monoclonal: negative reagent control that matches the isotype as the primary antibody. Additionally, the diluent used to manufacture a monoclonal primary antibody and isotypic negative control should contain the same ions. Diluents containing sodium or phosphate ions may change the sensitivity of some monoclonal antibodies.
  - Calculation:
    - Ig or total protein concentration of primary antibody divided by dilution factor of primary antibody = x
    - Ig or total protein concentration of negative reagent control divided by x = dilution factor of negative reagent control

Section 3 | Troubleshooting Chart

Tissue Specimen: Successful staining of tissue with an IHC marker is dependent on the type and preparation of the specimen. The chart below provides a convenient check list, as to [procedure at each step in the 'Total Test'.

Species: _____
(important to note in research studies).

Organ/tissue source: _____
Collection:

- ☐ Surgical specimen/biopsy
- ☐ Post-mortem specimen
- ☐ Fine needle aspirate
- ☐ Peripheral blood (include anti-coagulant)
- ☐ Brushing
- ☐ Biologic fluid
- ☐ Cell culture
- ☐ Other

Tissue preparation:

- ☐ Paraffin embedded
- ☐ Plastic embedded
- ☐ Cryostat section
- ☐ Cytospin
- ☐ Cell smear
- ☐ Mono-layer cultured cells
- ☐ Other

Tissue fixation:

- ☐ Type of fixative
- ☐ Total length of time in fixative, including during transport, grossing and on the tissue processor
- ☐ Size of specimen ; size of block; wheterh additional blocks are available if needed

  Tissue mounting:
- ☐ Slide mount
- ☐ Tissue thickness
- ☐ Gelatin, glue commercial adhesive or starch in the water bath
- ☐ Other

Blocking of endogenous components that may produce spurious staining.

Background staining is defined as unexpected or undesirable staining seen on the test or control tissue, which does not represent the target antigen. Frequent causes of background staining are endogenous enzyme activity and endogenous biotin.

Peroxidase is an enzyme of the oxido-reductase class that reacts with a substrate containing hydrogen peroxide as the electron acceptor. To block this activity, a variety of hydrogen peroxide reagents can be applied to cells producing this enzyme.

Alkaline phosphatase is an enzyme having various isoforms, which are produced in the leukocytes, liver, bone, intestine, placenta and Regan (carcinoma). Addition of levamisole to the chromogen/substrate will inhibit endogenous alkaline phosphatase activity, with the exception of the intestinal isoform. If necessary, this can be blocked with a weak acid wash, such as 0.03-0.5 N HCl.

Biotin, a B vitamin, may be protein-bound to tissue and can interfere with proper interpretation of staining patterns when using a streptavidin or avidin reagent. To block this binding, a biotin/avidin block.

Peroxidase block:

- ☐ 3% H_2O_2
- ☐ Methanol/H_2O_2
- ☐ Sodium azide
- ☐ Peroxidase Block (e.g. Dako Code S2003)
- ☐ Other

Alkaline phosphatase block:

- ☐ Levamisole
- ☐ 0.03 N HCl (not for use on cryostat tissue)
- ☐ Other

Biotin block:

- ☐ Biotin Block (e.g. Dako Code X0590)
- ☐ Other (e.g. skimmed milk)

Protein block:

- ☐ Protein Block (e.g. Dako Code X0909)
- ☐ Normal sera or Ig from host species of the secondary antibody
- ☐ Other

Section 4 | Specification Sheets

Below is an example of the information supplied in a typical Dako package insert for an IVD (in vitro diagnostic) concentrated antibody. The information and placement in the package insert will vary.

Information You Need to Know	Information Located on the Specification Sheet/Package Insert*	Comments
Regulatory Status of the Primary Antibody	Intended use ■ For in vitro diagnostic use	Indicates that a product meets the FDA requirements as a clinical diagnostic product. Likewise, a CE icon indicates the reagent meets European Union requirements.
Tissue Preparation	**Specimen preparation** Paraffin sections: The antibody can be used for labeling formalin-fixed, paraffin-embedded tissue sections. Tissue specimens should be cut into sections of approximately 4 µm. Pre-treatment: Pre-treatment of formalin-fixed, paraffin-embedded tissue sections with heat-induced epitope retrieval (HIER) is required. Optimal results are obtained by pretreating tissues with HIER using diluted EnVision™ FLEX Target Retrieval Solution, High pH (50x) (Codes K8000/K8004). Deparaffinization, rehydration and epitope retrieval can be performed in Dako PT Link (Code PT100/PT101). For details, please refer to PT Link User Guide. The tissue sections should not dry out during the treatment or during the following immunohistochemical staining procedure. For greater adherence of tissue sections to glass slides, the use of FLEX IHC Microscope Slides (Code K8020) is recommended. After staining the sections must be dehydrated, cleared and mounted using permanent mounting medium.	Indicates the type of specimen that was used during validation studies. In many cases this would include formalin-fixed tissue and frozen sections. Use of other fixatives requires validation by each individual laboratory. This section also indicates the optimal epitope retrieval procedure and warns against procedures that may destroy the epitope. Specimen preparation and staining procedure sections can and will change periodically, to reflect changes in technology. So remember to retain copies of each version of the reagent specification sheet. Version numbers are usually found on each page.
Choosing the Visualization System	**Staining procedure** Visualization: The recommended visualization system is EnVision™ FLEX, High pH (Code K8000/K8010) using a 20 minute incubation at room temperature. Follow the procedure enclosed with the selected visualization system(s). Automation: The antibody is well-suited for immunohistochemical staining using automated platforms, such as Dako Autostainer, Autostainer Plus and Autostainer Link as well as PT Link for pre-treatment.	Indicates the recommended visualization system to be used with the antibody. Conditions will differ if other detection systems are used. It also indicates that the antibody can be used for automated staining. NOTE: If your state regulatory agency requires written documentation that a reagent can be used for automated staining and this indication is not listed on the specification sheet, you may wish to contact the manufacturer's technical support group for further information.
Diluting the Primary Antibody	**Staining procedure** Dilution: The recommended dilution of Monoclonal Mouse Anti-Human PSMA, Clone 3E6, Code M3620, is 1:50. Dilute the antibody in Dako Antibody Diluent (Code S0809). Incubate pretreated tissue sections for 20 minutes at room temperature. These are guidelines only. Optimal conditions may vary depending on specimen and preparation method, and should be validated individually by each laboratory.	Includes a suggested dilution for the antibody and the recommended diluent. The dilution is a suggested starting point, but may require further optimization depending on specimen, preparation method, temperature of the laboratory or automated instrumentation.

* Examples included. The information given will vary depending on the individual antibodies. Always refer to the actual package insert for specific information

Information You Need to Know	Information Located on the Specification Sheet/Package Insert*	Comments
Controls	**Staining procedure** Controls: Positive and negative control tissues should be run simultaneously using the same protocol as the patient specimens. The positive control tissue should include prostate and the cells/structures should display reaction patterns as described for this tissue in the "Performance characteristics" section. Negative control: The recommended negative control reagent is Dako Negative Control, Mouse IgG1 (Code X0931), diluted to the same Ig concentration as the primary antibody. Unless the stability of the diluted antibody and negative control has been established in the actual staining procedure, dilute these reagents immediately prior to use. Positive and negative controls should be run simultaneously with patient specimens.	Use of a negative reagent control (ANP.22570) is no longer required by the College of American Pathologists (CAP), based on Clinical Laboratory Improvement Amendments (revised July 31 CLIA 2012), for each patient or patient block in a staining run when using polymer detection systems. The latest guidelines from CAP (5) leave it up to the individual laboratory to evaluate whether the selected detection method poses a negligible risk for non-specific staining that the negative reagent control for the polymer-based detection methods can be eliminated.
Positive Control Tissue	**Performance characteristics** Normal tissues: In prostate, glandular epithelial cells show a moderate to strong cytoplasmic and/or membranous staining reaction. Abnormal tissues: In 92/102 prostate adenocarcinoma, glandular epithelial cells showed a moderate to strong cytoplasmic and/or membranous staining reaction.	**CLIA 2003 Sec. 493.1273 (3)** Mandates that fluorescent and immunohistochemical stains must be checked for appropriate positive and negative reactivity each time they are used. Most IVD antibody specification sheet will list tissue that will exhibit positive and negative staining patterns in the Performance Characteristics section. NOTE: abnormal tissue will not necessarily be labeled. Both negative and positive tissue controls should be processed using the same fixation, embedding, mounting, drying, epitope retrieval and immunostaining protocols as the patient tissue.

Section 5 | Automated Platform Performance Checks

Information You Need to Know	Information Located at the Instrument	Comments
The right protocol has been applied to the slide	Right protocol ■ Find the location for the completed slides in the automation software and look up the slide ID of the slide in question and check that the applied protocol is the correct one	The information can be located in different places based on your automated solution. If the location is not known then contact your automated platform supplier

Information You Need to Know	Information Located at the Instrument	Comments
Target retrieval has been performed under the right conditions	**Target retrieval** Target retrieval solution: ■ Find the location of the specific data for the target retrieval procedure – this information can be a part of the slides' log file under completed slides ■ Check that the right target retrieval solution has been used. Sub-optimal results can be seen if a high pH target retrieval solution is used for an antibody that according to the specification sheet requires a low pH target retrieval solution. ■ Check that the target retrieval solution has been within the expiration limits when used for the slide Target retrieval temperature: ■ Check that the temperature has been held within the limits of the target retrieval equipment throughout the course of the target retrieval process. A too high temperature can lead to impaired morphology and "over retrieval" of the antigen epitopes. Low temperature can lead to inadequate retrieval of the epitopes and thereby reduced staining intensity or lack of stained epitopes. The temperature data for the slide can be located at different places in the instrument software based on which automated solution is used. It is recommended to consult the user guides for the automated platform or to contact the supplier. ■ High altitude installations need to provide information in the datalog that appropriate temperature was achieved Target retrieval time: ■ Find the location of the specific data for the target retrieval time. The target retrieval time for the slide can be located at different places in the automated platforms software based on which automated solution is used. It is recommended to consult the user guides for the automated platform to find the location or to contact the supplier. ■ Check that the time the slide actually received target retrieval is within the allowed limit for the assay performed ■ If positive control has been run for the assay on the slide in question an evaluation of the effect of any deviations can be made based on the positive control. Based on this evaluation it can be determined if the slide can be used even though not processed inside the allowed limits. Special care shall be taken if the time has been reduced because the level of positivity in the samples is not known and can differ from the positive control and thereby potentially result in a false negative result.	If any of the checks performed for the target retrieval step show irregularities it is recommended to re-run the sample and/or to get the automated equipment serviced by the manufacturer. If a failure of either use of the target retrieval reagents or the target retrieval platform has been identified, remember to search for other slides which potentially have been submitted to the same failure and perform a quality check of these related slides. Check of the instrument overall performance is not based on a single slide. However, if a given automated platform is the main denominator between failing slides then it should be considered to make a check of the automated platform and potentially get it serviced by the supplier.
LIS Protocols align with workstation test	Protocols from the server ■ Verify that correct protocols are received from the server ■ Find the appropriate test name for the protocol. Verify that this name is mapped in the list of IHC or ISH test protocols. Contact the vendor technical support or bioinformatics department for assistance.	Important to check this whenever new protocols or tests have been added to the server of the system. LIS may not automatically update and map new test protocols from the server.
Staining process has been performed under the right conditions	**Staining process** Reagents: ■ Find the location of the specific data for the executed protocol – this can be separate or a part of the slides' log file under completed slides. The location of the information can be looked up in the user guides for the automated platform or provided by the supplier. ■ Check that the right protocol including the right reagents have been used to stain the slide in question. Sub-optimal results may be seen if another reagent than the validated for the assay either by the laboratory itself or the supplier and the laboratory in combination is used. ■ Check that the right reagent volume has been applied according to the protocol ■ Check that all the reagents used have been within the expiration limits when used for the slide ■ Check that the label of the reagents used is actually in agreement with liquid in the bottles used on the automated platform. This can be done by looking at what the bottle previously has been used for in the bottle history (can be located different places dependent of automated platform used). If the bottle history shows successful use of the reagent prior to this then the right reagent is in the bottle.	If you use reagents that you dilute from concentrate then it is important to check that the dilution has been done correctly. This can be done by validating the new dilution against a previous dilution still within the expiry limits. It is recommended that these checks are done to eliminate other variables like different automated platform or other reagents in the process. It is recommended that controls are applied on every slide to ensure that it has received the exact same treatment as the sample being evaluated which supports the trouble shooting for the samples to a higher degree than a separate run or daily control will do.

Information You Need to Know	Information Located at the Instrument	Comments
	■ Check whether the reagent has been stored according to recommendation from the supplier ■ Check that the reagent volume usages have been adequate compared to the calculated use. This can be done by checking the bottle history together with the weight of an empty container and compare this to the container used making it possible to estimate the consumption. Staining temperature: ■ If temperature control is used during the staining process then check that the temperature has been held within the limits of the staining process. The location of the information can be found in the user guides for the automated platform or provided by the supplier. ■ Check that the operating conditions for the automated platform are within specifications. The specified operating conditions for automated platforms are listed in the user guides. Incubation time: ■ Find the location of the executed incubation times for the slide in question. This location can vary based on which automated platform is used. The location of this information is accessible either from the user guides or can be provided by the supplier. ■ Check that the incubation times the slide actually received are within the allowed limits for the assay performed. Variation in incubation time can result in variation in staining results. Too long incubation time can potentially lead to increased level of background staining which can influence the interpretation of the slide. Too short incubation times can lead to false negative results. Wash: ■ Find the location of the executed wash incubation times for the slide in question. This location can vary based on which automated platform has been used. The location of this information is accessible either from the user guides or can be provided by the supplier. ■ Check that the incubation time the slide actually received in the wash steps are within the allowed limits for the assay performed. Wide variation in wash times can be allowed for most assays. However, too short wash time potentially can result in unwanted specific as well as non-specific background due to inadequate wash. Extensive wash times can for special assays lead to reduced intensity. ■ Check that the wash buffer volume has been within the acceptable limits. How this is done can be different based on the automated platform used. Most platforms have estimated buffer consumption per run which can be checked against the actual usages. Furthermore, description of how to check the wash volume per slide may be provided in the user guides or can be requested from the supplier. ■ Check that the wash buffer used is the buffer specified for the assay or has been validated for the assay by the laboratory ■ Check that the wash buffer used for the slide has been within the expiry limits when used for the slide in question General: ■ If a positive control has been run for the assay on the slide in question an evaluation of the effect of any deviations can be made based on the positive control. Based on this evaluation it can be determined if the slide can be used even though not processed inside the allowed limits. Special care shall be taken if the time has been reduced because the level of positivity in the samples is not known and can differ from the positive control and thereby potentially result in a false negative result. ■ Check of leveling of the slide during the staining process. How this is done will vary based on the automated platform used. The information can be obtained either from the user guides for the automated platform or requested from the the supplier. It is important to check the level of the slide as a slide which is not in level during staining can lead to the area of interest not receiving the necessary reagents. This will result in inconsistent staining.	If problems with the automated platform is indentified it is recommended to check other samples processed in the same period on the same platform or platforms in order to identify potential relations. To confirm or discharge the automated platform as the reason to problems with the staining quality it is a good idea to search for related samples e.g. other samples treated on the same automated platform run or in the same slide position, this can help lead in the right direction when doing the troubleshooting. If problems with the automated platform is identified, a service of the instrument is recommended

Information You Need to Know	Information Located at the Instrument
Automated IHC staining platform has been performing as expected	**Automated IHC staining platform** Reagent volume: ■ Make sure that the volume your automated platform is set to use is adequate to cover the entire area of your sample over the total duration of the incubation time. It is important to take into account the potential evaporation of the reagent over time. Drying out of the tissue during the staining process can result in staining effects including no staining, inconsistent staining, extensive background and other staining artifacts Contamination: ■ Contamination of the probe on the automated platform can lead to mixing of reagents in the probe and/or on the slide. This can lead to false positive staining or background on the slide. The inclusion of negative tissue controls can help identify if a contamination has taken place. A contamination of the visualization system in the substrate-chromogen will not be identified by the negative control as it will be present both on the positive control and the negative control. In order to identify this contamination it is necessary to make an investigational test where the first dispense of substrate chromogen can be removed and examined by mass spectrometry or ELISA for small quantities of visualization component. ■ Contamination with bacterial and/or fungal growth can be seen in automated IHC platforms when the recommended maintenance schedule is not followed and/or some parts has been defective which increase the risk of bacterial and/or fungal growth. Inspection of the visual parts of the instrument as well as keeping the maintenance schedule can prevent the contamination from happening. If growth is expected normal microbiological methods can be used to determine the presence of both bacteria and fungus. After identification the automated platform has to be cleaned according to specifications listed in the user guides or the supplier can be contacted for advice on how to clean. ■ Contamination caused by inadequate wash of the automated platforms probe. After the probe aspirates reagent it has to be washed before transferred to another reagent bottle. If this wash is not adequate then the second bottle can be contaminated with reagent from the previous bottle. This will be identified as an unspecific reaction for a given marker e.g. CD20 staining in the nuclei if the first aspiration wash from Ki67. This contamination can be confirmed by having the probe go into a bottle of wash buffer or other neutral fluids after the first aspiration and then measure the content of the previous reagent by either mass spectrometry or ELISA. ■ Contamination of the tissue by the probe dropping a drop of unrelated reagent onto the slide. This will be recognized as a false positive reaction either being in the wrong structure or wrong location. Use of positive tissue controls including multiple organ types on each slide will make it possible to identify contamination of this origin. Waste separation: ■ Automated platform separates hazardous from non-hazardous waste. Failure of this separation does not impact the staining process and thereby should not influence the staining result. Bulk fluid supply: ■ The automated platforms normally have a function which makes it possible to check whether the supply of bulk fluids is working adequately. This check can be a prime of the bulk fluid trough the system securing that there are no leakages or clots preventing the fluid from flowing. Check of the bulk fluid supply is described in the user guides or can be requested from the supplier. General: ■ If in doubt about the performance of the instrument, thorough observation of the automated platform during the staining process can give an indication of whether the individual steps is being executed as expected. ■ Check that the instrument is closed correctly as the performance depends on the surrounding conditions for optimal staining. Improper closing can result in inability to start or create staining conditions which are sub-optimal do to the air getting into the instrument through the open cabinet. ■ Instrumentation should be installed away from direct sunlight. Sunlight can affect the staining conditions and viability of reagents.

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